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Expression Profile of Intraovarian Genes in Ovary Tissues at Follicular and Luteal Phases in Holstein Cattle[#]

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ABSTRACT

The aim of the present study is to determine comparatively expression levels of the *BMP15*, *TGFB1*, *TGFB2* and *GDF9* genes in the preovulatory follicle and corpus luteum tissue of Holstein cattle. For this purpose, primarily the tissues were examined by immunohistochemical staining. Later on, follicular fluid was analyzed by ELISA test and estradiol and progesterone levels were determined. Finally, expression levels of the related genes were determined between the groups by qRT-PCR. Immunohistochemical staining revealed that estrogen receptor alpha and progesterone receptor immunoreactivities were intensely present in preovulatory follicles, estrogen alpha receptor immunoreactivity was very slight and progesterone receptors were similar to positivity in preovulatory follicles in corpus luteum. Furthermore, estradiol level was high in preovulatory follicles and progesterone level was high in corpus luteum. The levels of mRNA transcripts of the *TGFB1* and *TGFB2* genes in the preovulatory follicles were statistically higher than the corpus luteum ($p<0.01$, $p<0.05$, respectively), but there was no statistically significant difference between the groups in the mRNA transcript levels of the *BMP15* and *GDF9* genes ($p>0.05$). As a result, it is thought that differential expression of intraovarian genes may be associated with differences in follicular dynamics and gene expression levels within the cell population of ovarian tissue, so this situation may result in follicular and luteal phase.
Key Words: Corpus luteum, holstein, intraovarian genes, preovulatory follicle, qRT-PCR.

Holştayn Sığırlarında Foliküler ve Luteal Fazdaki Ovaryum Dokularında İntraovarian Genlerin Ekspresyon Profili

ÖZ

Bu çalışmanın amacı, Holştayn sığırlarına ait preovülatör folikül ve korpus luteum dokularında *BMP15*, *TGFB1*, *TGFB2* ve *GDF9* genlerinin ekspresyon seviyelerini karşılaştırılmalı olarak belirlemektir. Bu amaç için, öncelikle dokular immunohistokimyasal boyama ile incelendi. Daha sonra foliküler sıvılar ELISA testiyle incelenerek östradiol ve progesteron seviyeleri belirlendi. Son olarak qRT-PCR ile gruplar arasında ilgili genlere ait ekspresyon seviyeleri tespit edildi. İmmunohistokimyasal boyama sonucunda preovülatör foliküllerde yoğun miktarda östrojen reseptör alfa ve progesteron reseptör immunpozitifliklerine, korpus luteum da çok hafif düzeyde östrogen alfa reseptör immunpozitifliğine, progesteron reseptörlerinin ise preovülatör foliküllerdeki pozitiflik düzeyine yakın olduğu belirlendi. Östradiol seviyesi, preovulatör foliküllerde yüksek, progesteron seviyesi ise korpus luteumda yüksek olarak bulundu. Preovülatör foliküllerdeki *TGFB1* ve *TGFB2* genlerine ait mRNA transkript seviyesi korpus luteuma göre istatistiksel olarak daha yüksek bulundu ($p<0.01$, $p<0.05$, sırasıyla), ancak gruplar arasında *BMP15* ve *GDF9* genlerine ait mRNA transkript seviyesinde istatistiksel olarak bir fark gözlenmedi ($p>0.05$). Sonuç olarak intraovarian genlerin farklı ekspresyonunun, ovaryum dokusunu oluşturan hücre popülasyonu içinde folikül dinamikleri ve gen ekspresyon seviyelerinin farklılıklarıyla ilişkili olabileceğini ve bu durumun da foliküler ve luteal dönemin sonucu olarak ortaya çıkabileceği düşünülmektedir.
Anahtar Kelimeler: Korpus luteum, holştayn, , intraovarian genler, preovulatör folikül, qRT-PCR.

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INTRODUCTION

Ovary activity and hormones are very important in terms of pubertal development and normal reproductive performance (Bliss et al. 2010). Enhancement of reproductive performance in cattle breeding, has great significance due to its effect on farm productivity as well as an effective herd management and profitable production from the industrial aspect (Ball and Peters 2004, Burrow 2012). The periodic growth in cattle ovaries is regulated through FSH which is the hormone stimulating follicle (Donadeu and Pedersen 2008). Follicle phase is the first phase of growth in the ovarium. In the follicular phase, the largest follicle continues to grow while the other small follicles suffer atresia. The follicle continuing to grow can be converted to corpus luteum subsequent to ovulation. There are millions of genes responsible for the developmental phases of ovaries in cattle. Numerous genes have been identified at the transformation from luteal phase to follicle selection, maturation, and follicle phases as a result of transcriptome analyses (Rao et al. 2008). Determination of gene expressions engaged in the development of follicles as well as interactions between these genes may produce practical results in the context of animal breeding.
Estrogen is an important steroid hormone which regulates steroidogenesis and folliculogenesis (Merk et al. 1972, Drummond and Findlay 1999). There are two important estrogen receptors as estrogen receptor alpha (ER-a) and estrogen receptor beta (ER-b). The foregoing receptors exist in follicles, interstitial tissue, germinal epithelium during different phases of development (Huias-Stasiak and Gawron 2007, Panoulis et al. 2015). Progesterone has an important role in the regulation of the reproductive cycle. Progesterone binds to intracellular progesterone receptors in target tissues and becomes active subsequently (Delman and Eurell 1988). Progesterone also has two forms as A and B like estrogen receptors (Graham and Clarke 1997, Wolfer et al. 2016).

Regulation of ovarian activity is a complex process comprising FSH and LH receptors, ovarian steroids as well as intra- variant factors (Van den Hurk and Zhao 2005). The most important in intraovarian factors include bone morphogenetic proteins (*BMPs*) 6, 7 and 15 (*BMP6, BMP 7* and *BMP15*), ovarian somatic cells and transforming growth factor-beta (*TGFB*) expressed specifically by oocytes during the folliculogenesis phase (*TGFB1* and *TGFB2*) are members of the superfamily (Knight and Glister 2006, Otsuka 2010). The cited genes regulate ovarian follicular development at the molecular level in addition to affecting fertility (Corduk et al. 2012, Nagashima et al. 2011, Paradis et al. 2009). Furthermore, the growth differentiation factor 9 (*GDF9*) gene expression is important for both humans and animals as regards oocyte competence and fertility (Barros et al. 2012).

There is not sufficient information with respect to *BMP15*, *TGFB1*, *TGFB2,* and *GDF9* gene expressions in ovaries at follicular, and luteal phases of Holstein breed. The objective of this study is to determine *BMP15*, *TGFB1*, *TGFB2* and *GDF9* gene expressions in ovaries at follicles and luteal phases. The different expression levels of these genes will furnish useful information as regard the determination of the role of these genes in follicular development as well as their molecular mechanisms.

MATERIAL and METHOD

Material

The animal material utilized in the study comprised 20 specimens of ovaries from Holstein cattle following the slaughter thereof. Totally 20 Holstein cows (n=20), around 5 years old, were selected to obtain the ovary tissue samples. The selection criteria of cows are multiparous, lactating (late lactation stage), and body condition score of 3.0 to ≤ 3.50. The ovaries collected following the slaughter carried out at Oral Meat Integrated Facilities in Erzurum province were sterilized by 70% ethanol. The preovulatory follicle (POF) and corpus luteum (CL) tissue samples were measured by virtue of Vernier caliper. The samples of received POF were about 11.18 ± 1.8 mm while received CL samples were about 14.32 ± 1.57 mm. Subsequently, tissue samples were then quickly frozen in liquid nitrogen and stored at -80 ^{0}C.

Method

Immunohistochemical staining

The tissues fixed in neutral formaldehyde solution during 1 day were washed with tap water. The tissues were placed in paraffin blocks after the alcohol-xylol follow-up process. After the deparaffinization of the tissues placed on the polylysine-coated slides was conducted, the cells were incubated in 3% H_2O_2 for 10 minutes to inactivate endogenous peroxidase activity and washed in PBS. Subsequently, they were kept in the antigen retrieval solution for 10 min at 500W in order to remove the antigens in the tissues and then washed in PBS. Protein block solution was added with a view to preventing nonspecific bindings and then they were washed in

PBS. Progesterone R / NR3C3 Antibody (Alpha PR6) (NB120-2765) and Estrogen Receptor alpha Polyclonal Antibody (PA5-16476) antibodies were applied as primer antibody at 1/100 dilution ratios to the sections washed with PBS. Subsequently, the procedure indicated in Expose mouse and rabbit specific HRP/DAB detection IHC kit (Abcam: ab80436) was followed. 3,3' diaminobenzidine chromogen was employed and stained with hematoxylin as contrast. Positive cells were examined by virtue of a light microscope at 20x magnification.

ELISA Test

Estradiol and progesterone concentrations in follicular fluid were measured to determine progesterone and estrogen active follicles. 7β-Estradiol high sensitivity ELISA kit (Enzo Life Sciences, UK) was used for estradiol while Progesterone Competitive ELISA Kit (Thermo Fisher Scientific, USA) kit was used for progesterone. Concentration measurement was performed according to the procedure given in the ELISA kits. (Standard curve, 15.6-1,000 pg / ml E2 and 50 pg / mL-3,200 pg / mL progesterone). In addition, we calculated the recovery of ELISA results (Table 1 and Table 2).

Total RNA Isolation

Total RNA isolation was realized from the collected tissue samples through the utilization of Trizol (Invitrogen, USA). Total RNA isolation was realized in line with the kit's procedure. Following the total RNA isolation, the RNA concentration was measured by virtue of NanoDrop (Epoch Microplate Spectrophotometer, USA). RNAs were run in a 1.5% agarose gel in 1XTBE solution for one hour at 80 volts with a view to control total RNA quality and visualized by gel imaging system (Bio-Rad Gel Doc XR+) and their RNA quality was determined (Fig. S1).

DNase I treatment and cDNA Synthesis

DNase I (Thermo Scientific, USA) was employed against DNA contamination in isolated RNA samples. Dnaz I treatment was performed in line with the protocol provided in the kit. Subsequently, 1 µg was taken from these RNAs and cDNA was synthesized through utilization of the miScript Reverse Transcription Kit (Qiagen, Germany) in line with the protocol provided. The purity and quantity of the obtained cDNAs were measured by virtue of spectrophotometer (Epoch Microplate Spectrophotometer, USA), and the cDNAs were diluted at the same ratios. Subsequently, the cDNA samples were stored at -20 ^{0}C for utilization in Real Time PCR studies.

Real time PCR

qRT-PCR was performed through utilization of the CFX96 BioRad device in order to measure the mRNA transcript levels of the *BMP15*, *TGFB1*, *TGFB2* and *GDF9* genes. The GAPDH gene was employed for internal control. Master mix content created in real time PCR experiments is as follows: Syber Green 2X Rox Dye Master mix (Qiagen Germany), forward and reverse primers designed for genes, cDNAs as template and nuclease-free water. The samples were analyzed in Real Time device following the preparation of master mixes and the obtained Ct values were calculated according to $2^{-DeltaDeltaCt}$ method and expression levels of the respective genes were determined (Livak and Schmittgen 2001). Reaction conditions and primer sequences of the genes are shown in Table 3. The primer sequences were received from a previously conducted study (Weller et al. 2016).

Statistical analysis

IBM SPSS 20 program was employed for statistical analysis. Statistical differences of mRNA transcript levels of *BMP15*, *TGFB1*, *TGFB2* and *GDF9* genes were analyzed through utilization of t-test method while statistical differences of Estradiol and progesterone concentration (pg/ml) was determined through utilization of one-way analysis of variance (ANOVA). Relative mRNA expression graphics and estradiol and progesterone concentration graphics were generated by using Graph pad prism software Inc., (Version 7.0, California, USA). qRT-PCR results were expressed as mean ± SEM (standard error of the mean). For statistical comparisons, probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$were accepted as statistically significant.

RESULTS

Immunohistochemical Examination

As a result of the immunohistochemical staining, intense amounts of estrogen receptor alpha (Fig. 1A) and progesterone receptor immunopositivity (Fig. 1B) were observed in the ovaries thought to be at follicular phases. Estrogen receptor alpha and progesterone receptor immunopositives at the follicular phase were observed in the theca interna (arrow) and theca externa (arrowhead) cells. Estrogen alpha receptor was found to be at very mild level at the luteal phase in the lutein cells (arrow) due to the decrease in immunopositivity (Fig.1C) In progesterone receptors, it was determined to have the close positivity level in the lutein cells' (arrow) follicular phase (Fig. 1D).

Estradiol and Progesterone Levels in POF and CL

ELISA test was applied to the collected preovulatory follicles and corpus luteums and they were analyzed in terms of estradiol and progesterone. Estradiol levels were higher in ≥10 mm preovulatory follicles (predominantly sized follicles classified as healthy) as expected, (Fig. 2A). Progesterone levels were relatively high in the corpus luteum (Fig. 2B).

Transcriptional Analysis

Expression levels of *BMP15*, *TGFB1*, *TGFB2* and *GDF9* genes in POF and CL were measured by virtue of qRT-PCR. mRNA transcript levels of *TGFB1* and *TGFB2* genes in POF were found statistically higher compared to CL (Figs. 3A and 3B) (p <0.01, p <0.05, respectively), but no statistically significant difference was observed in mRNA transcript level of *BMP15* and GDF 9 genes between groups (Figs. 4A and 4B) (p> 0.05).

Table 1. Recovery rate for estradiol

Low Samples %	High Samples %	Expected Conc. (pg/ml)	Observed Conc. (pg/ml)	% Recovery
80	20	457.3	504.7	111.9
60	40	655.5	717.2	108.2
40	60	1035.4	1082.8	104.5
20	80	1288.9	1345.1	103.5
			Mean recovery	**108.5%**

Table 2. Recovery rate for progesterone

Low Samples %	High Samples %	Expected Conc. (pg/ml)	Observed Conc. (pg/ml)	% Recovery
80	20	651.2	699.3	105.1
60	40	962.1	1024.4	104.3
40	60	1253.8	1324.8	103.9
20	80	1551.7	1602.7	102.2
			Mean recovery	**105.4%**

Table 3. Primer sequences of GAPDH, *TGFB1*, *TGFB2*, *BMP15* ve *GDF9* genes

Primer	Sequences (5'-3')	Annealing ^{0}C	Base Pair	Accession Number	Reaction Conditions
GAPDH	F: GATGCTGGTGCTGAGTATGT R: GCAGAAGGTGCAGAGATGAT	58	113	NM_001034034.2	94°C 15 s / 58°C 30 s / 72°C 30 s (40 cycles)
TGFB1	F: TGCTTCAGCTCCACAGAAA R: GTATCCAGGCTCCAGATGTAAG	58	149	NM_001166068.1	94°C 15 s / 58°C 30 s / 72°C 30 s (40 cycles)
TGFB2	F: CACGAATGGCTCCACCATAA R: AGCGTGCTTCTAGTTCTTCAC	58	127	NM_001113252.1	94°C 15 s / 58°C 30 s / 72°C 30 s (40 cycles)
BMP15	F: GTAGTGAGGTTCGTGAGTTCTG R: TAGGGAGAGGTTTGGTCTTCT	58	111	NM_001031752.1	94°C 15 s / 58°C 30 s / 72°C 30 s (40 cycles)
GDF9	F: GCATTCCCTCCACCCTAAA R: GGTGACGGGACAATCTTACA	58	113	NM_174681.2	94°C 15 s / 58°C 30 s / 72°C 30 s (40 cycles)

*Referance gene (internal control).

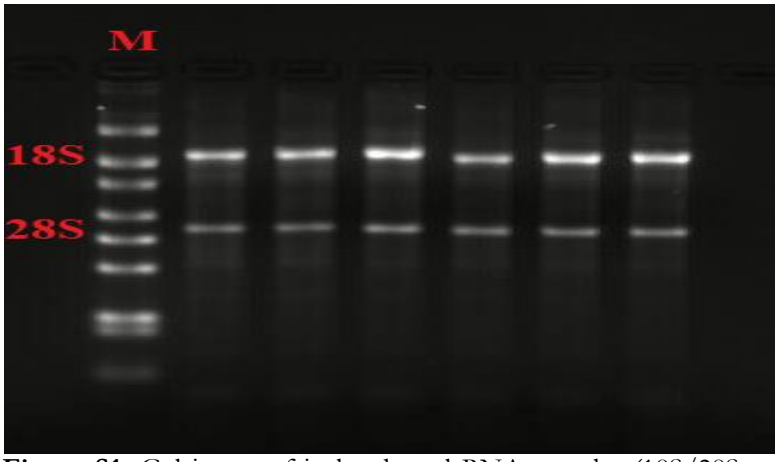

Figure S1. Gel image of isolated total RNA samples (18S/28S RNA)

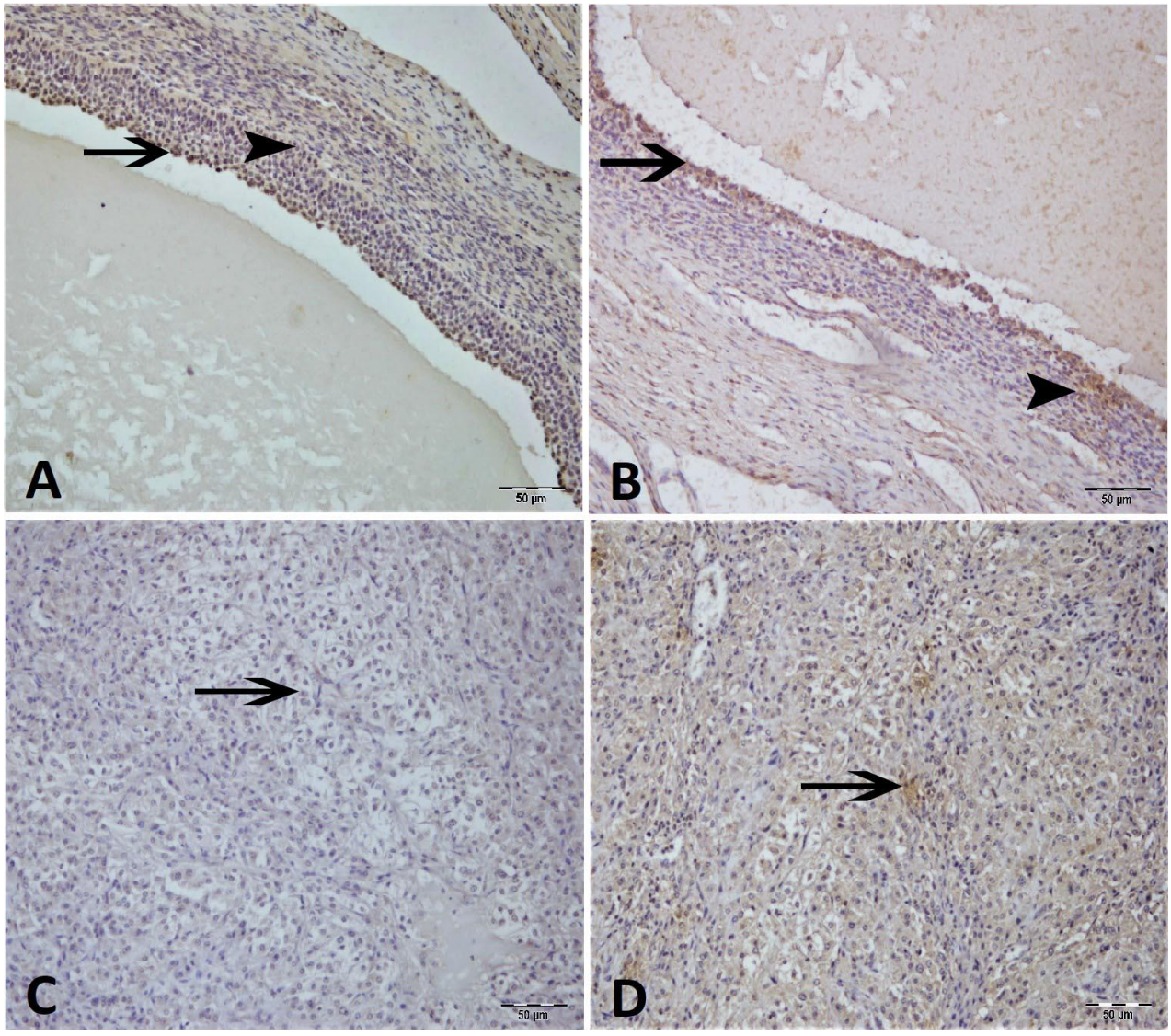

Figure 1. A) Follicular stage; intense estrogen receptor alpha immunopositivity in Teka externa (arrowhead) and teka interna (arrow) cells. B) Follicular stage; Immunopositivity of progesterone receptors in Teka externa (arrow head) and teka interna (arrow) cells. C) Luteal stage; estrogen receptor alpha immunopositivity at very mild level in lutein cells (arrow). D) Luteal stage; progesterone receptor immunopositivity in lutein cells (arrow)

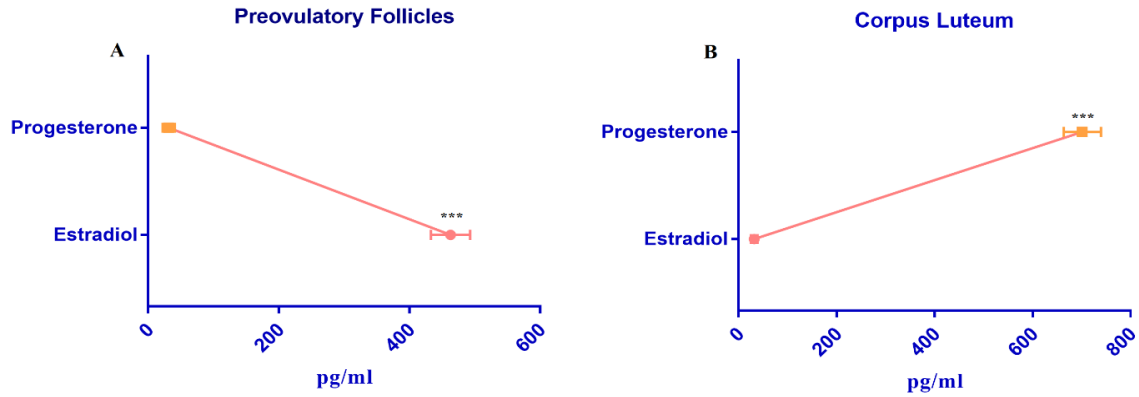

Figure 2. Estradiol / Progesterone levels in the preovulatory follicle and corpus luteum (pg/ml)

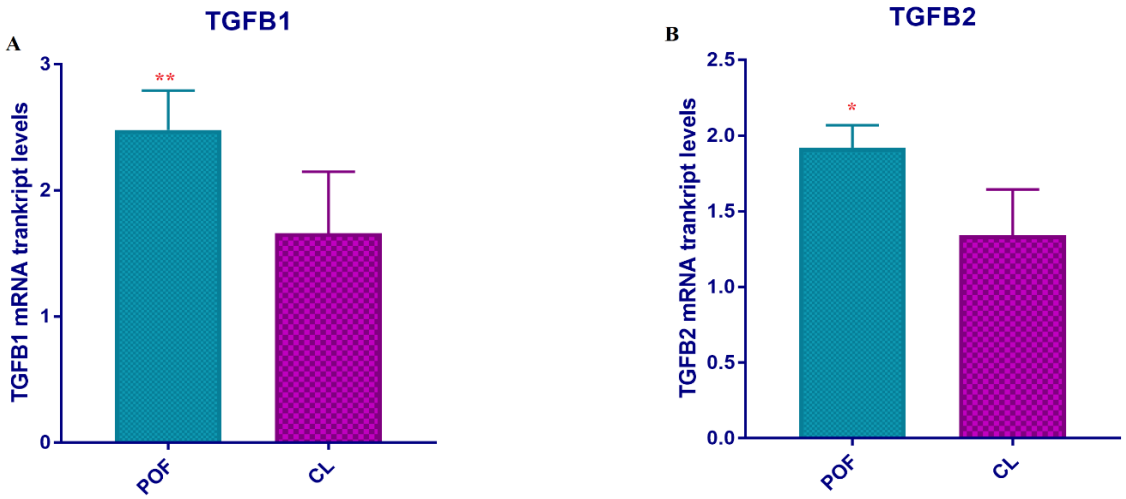

Figure 3. mRNA transcript levels of the *TGFB1* and *TGFB2* genes in the preovulatory follicle and corpus luteum tissues. Values represent the mean ± SD of 3 independent samples; The error bars show the standard deviation. Statistical significance (* p<0.05, ** p <0.01) was analyzed by One Way ANOVA. A) Represent the relative mRNA expression levels of the *TGFB1* gene. B) Represent the relative mRNA expression levels of the *TGFB2* gene

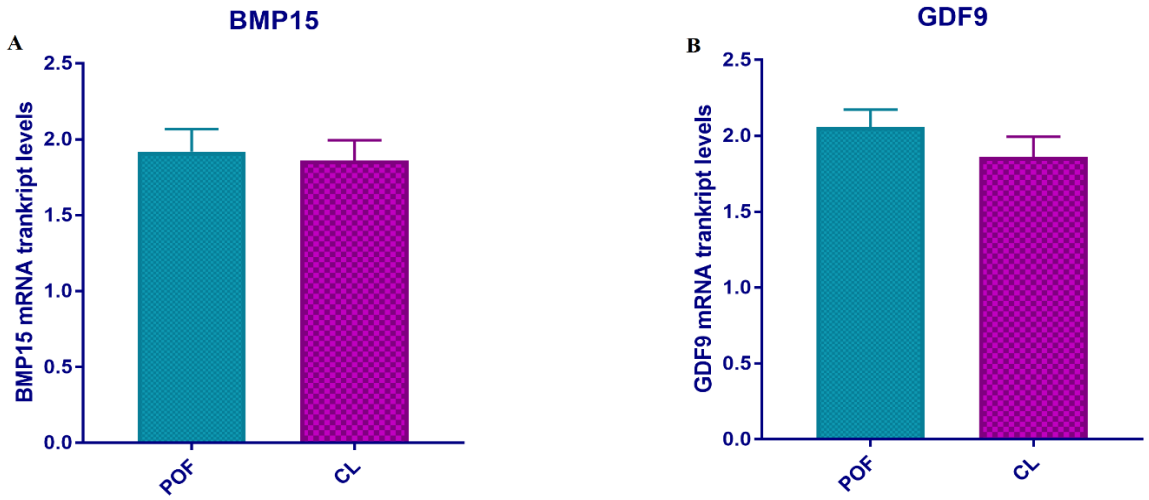

Figure 4. mRNA transcript levels of the *BMP15* and *GDF9* genes in the preovulatory follicle and corpus luteum tissues. Values represent the mean ± SD of 3 independent samples; The error bars show the standard deviation. Statistical significance was analyzed by One Way ANOVA. A) Represent the relative mRNA expression levels of the *BMP15* gene. B) Represent the relative mRNA expression levels of the *GDF9* gene

DISCUSSION

This study reveals different expression levels of the *BMP15*, *TGFB1*, *TGFB2* and *GDF9* genes in the preovulatory follicle and corpus luteum. FSH, LH, ovarian steroids and intra-orbital factors have a role in regulation of ovarian activity (Van den Hurk and Zhao 2005). The genes of the TGFB superfamily, such as *GDF9*, *BMP15*, *TGFB1* and *TGFB2*, and the cell receptors thereof are known to be paracrine and autocrine modulators of ovarian functions and fertility (Knight and Glister 2006, Otsuka 2010, Corduk et al. 2012, Nagashima et al. 2011, Paradis et al. 2009). The findings we have obtained from this study is in compliance with current theories.
Berisha (2002) have examined the expression of estrogen and progesterone receptors in the ovaries of cattle during the estrous cycle period in a study conducted thereby. In this study, they have observed the fact that ERalpha receptors increased in granular cells during the follicular development in techa interna cells towards the end of the follicular phase. They have reported that the ERalpha receptors became at the highest level at the onset of the luteal phase, but decreased towards the middle and the end of the period. It was observed that there was no significant change in progesterone receptivity during the estrous cycle in the same study. Estrogen alpha and progesterone receptors were observed intensely in ovaries at the follicular phase in the present study. In ovaries at the luteal phase, estrogen receptor alpha levels decreased and progesterone receptor levels were found to be at the same level. The decrease in estrogen receptors at the luteal phase suggests that the cycle is at the middle or late phase of the luteal phase. As a result, it was observed that both receptors were intensified at the follicular phase, and estrogen receptor alpha decreased and progesterone receptor levels were at the same level at the middle or late periods of the luteal phase.

BMPs have an important role in the regulation of follicle development, ovulation, and KL morphogenesis (Otsuka et al. 2011, Shimasaki et al. 2004). Weller (2016) revealed that there was no change in the expression levels of *BMP15* and *GDF9* genes in follicular and luteal ovarian tissues of Bos indicus cattle. Similarly, there was no statistical difference in the gene expressions of *BMP15* and *GDF9* in POF and KL in this study. This result shows that the *BMP15* and *GDF9* genes are expressed without change in follicule development (in POF and KL processes) in mammals. This suggests that *BMP15* and *GDF9* genes actively have a role in molecular mechanisms with both follicular and luteal development.

Transforming growth factor-beta isoforms (*TGFB1* and *TGFB2*) are known as multifunctional regulatory molecules because they stimulate and inhibit proliferation, differentiation, and other critical cell functions according to type of ovarian cells, stage and other growth factors (Lee et al. 2001, Hanukoglu 1992, Juengel and McNatty 2005). *TGFB1* in cattle and *TGFB1* and *TGFB2* in sheep have an inhibitory effect on the proliferation of granulosa cells (Saragueta et al. 2002, Gilchrist et al. 2003). *TGFB1* mRNA and protein expression in cattle granulosa cells decreases during the progression of folliculogenesis (Juengel et al. 2004, Matiller et al. 2014, Farberov and Meidan 2016). Furthermore, in cattle, *TGFB1* is expressed in granulosa cells in the earliest phases of development (early preantral and early antral follicle) while it is expressed less in larger follicles (Oullette et al. 2005). In this study, we showed that *TGFB1* and *TGFB2* genes are expressed more in preovulatory follicles. This situation suggests us that inhibition of proliferation induced by *TGFB1* may make the cell more susceptible to FSH and causes differentiation of the granulosa cells. Moreover, this result is also consistent with previous studies (Oullette et al. 2005, Saragueta et al. 2002, Gilchrist et al. 2003, Farberov and Meidan 2016).

CONCLUSION

Briefly, our results suggest that differential expression of intraovarian genes may be related to differences in follicular dynamics and gene expression levels within the cell population which form the ovarian tissue. This result could occur as a result of the follicular and luteal phases. The determination of expression levels of the genes in the preovulatory follicle and the corpus luteum in cattle in a comparative way will furnish a source for studies to be conducted in order to determine the molecular mechanisms of these genes.

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